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Effect of conjugated linoleic acid mixture supplemented daily after carcinogen application on linoleic and arachidonic acid metabolites in rat serum and induced tumours

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ABSTRACT

Conjugated linoleic acid (CLA) is thought to exert anticarcinogenic, antiatherogenic, anti-inflammatory and weight loss effects. The impact on eicosanoid biosynthesis may be one of the mechanisms of its action. The aim of this study was to establish whether CLA mixture supplemented daily after administration of carcinogen (7, 12-dimethylbenz[a]anthracene, DMBA) influenced the concentration of linoleic and arachidonic acid metabolites: 13- or 9-hydroxyoctadecadienoic acids (13-, 9-HODE) and 15-, 12- or 5-hydroxyeicosatetraenoic acids (15-, 12- or 5-HETE) and prostaglandin E_2 (PGE_2) in rat serum and DMBA-induced tumours. The correlations between polyunsaturated fatty acids (PUFA) and HETE and HODE contents in serum were also investigated.
Female Sprague–Dawley rats divided into three groups according to the diet (1% Bio-C.L.A., 2% Bio-C.L.A. and plant oil in the control group) were used in the study. On the 50th day of life some of the animals in every dietary group were administered DMBA to induce tumours. Since that day, the rats were fed one of the above-mentioned diets. After 15 weeks the animals were sacrificed and blood and tumours were collected. HETE and HODE were extracted using a solid-phase extraction (SPE) method on C18 columns and analysed with LC-MS/MS.
The results of our study showed that CLA daily supplementation after carcinogen administration influence LA and AA metabolite levels in serum and tumours. However, the ratios of eicosanoids having opposite effects (e.g. 12-HETE/15-HETE), not concentrations of particular compounds, appear to be better indicators of pathological processes.

1. Introduction

Conjugated linoleic acid (CLA) is a group of naturally occurring fatty acids synthesised from linoleic acid by bacteria present in alimentary tract of ruminant animals or as a result of endogenous conversion of transvaccenic acid by Δ9-desaturase in tissues, especially the mammary glands [1]. CLA may be also synthetically produced by partial hydrogenation or alkali isomerisation of linoleic acid or oils rich in linoleic acid (e.g. sunflower or safflower oils) [2,3]. That pathway is used for the industrial production of commonly available CLA preparations. CLA is a mixture of positional and geometric isomers of linoleic acid (LA) with double bonds between carbon atoms 7 and 9, 8 and 10, 9 and 11, 10 and 12 or 11 and 13. They occur in a cis or trans configuration. The most prevalent form is the cis-9,trans-11 CLA isomer found in ruminant-derived foods like milk, cheese and meat [4]. The other active isomer is trans-10,cis-12 CLA, present only in trace amounts in animal-derived foods. However, it was found in equal proportions with cis-9, trans-11 CLA in commercial CLA supplements [4]. As Yu et al. indicated, these supplements may contain different concentrations of total CLA, its two main active isomers and fatty acid profile [5]. That may be due to various compositions of fatty acids in the source plant oil used to CLA synthesis and to conditions of isomerisation reactions [5].

CLA is thought to exert beneficial effects in atherosclerosis prevention [6], obesity reduction and cancer prevention [7,8]. Presently, the mechanisms of its anti-carcinogenic action are being researched. An impact of CLA on eicosanoid biosynthesis is thought to be one of them.

Eicosanoids are local, biologically active metabolites of 20-carbon polyunsaturated fatty acids — arachidonic acid (AA), dihomo-gamma-linolenic acid (DGLA), and eicosapentaenoic acid (EPA). They are synthesised by their oxidation by cyclooxygenase (COX) and lipoxygenases (LOX). Arachidonic acid derivatives synthesised by COX pathway belong to the best characterised compounds. They

Abbreviations: AA, arachidonic acid; CLA, conjugated linoleic acid; COX, cyclooxygenase; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DMBA, 7,12-dimethylbenz[a]anthracene; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LA, linoleic acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LOX, lipoxygenase; PUFA, polyunsaturated fatty acids; SEM, standard error of mean; TXA_2, thromboxane A_2

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include prostaglandin E_2 (PGE_2), prostacyclin or thromboxane A_2 (TXA_2). PGE_2 appears to be a pro-inflammatory mediator and was synthesised by some tumours [9]. Its elevated levels were noted in tumours of digestive tract and in blood collected from vessels draining a tumour area [10]. PGE_2 production correlated with fatty acid content in the diet and increased in animals fed a diet rich in linoleic acid. In contrast to COX metabolites, hydroxyeicosatetraenoic acids (HETE), synthesised from arachidonic acid by LOX were initially considered unimportant compounds without biological activity. Since different LOX isoforms are involved in arachidonic acid metabolism, three main isomers of HETE are generated: 5-, 12- and 15-hydroxyeicosatetraenoic acids (5-, 12-and 15-HETE). Nowadays they are thought to participate in a number of pathological processes, such as inflammation, atherosclerosis, hypertension or cancer [11]. 5- and 12-HETE are involved in tumour development [12]. 12-HETE may enhance tumour cell adhesion to endothelium, stimulate their proliferation, motility and angiogenesis and as a result plays a critical role in metastasis [13]. Exogenous 5-HETE stimulates the proliferation and growth of prostate, breast and lung cancer cells and acts as a survival factor [11,14]. The inhibition of its synthesis by nordihydroguaiaretic acid (NDGA), AA-861, MK-886 and Zileuton, which are 5-LOX inhibitors, resulted in decreased proliferation and enhanced apoptosis of cancer cells. Contrary to pro-cancerogenic 5- and 12-HETE, 15-HETE appears to have protective and anti-tumourigenic activity. It activates peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear transcription factor involved in epithelial differentiation and the arrest of cell growth. As a result, 15-HETE inhibits proliferation and induces apoptosis of prostate carcinoma or colorectal cancer cells [15,16]. Besides 15-HETE, 13-hydroxyoctadecadienoic acid (13-HODE), linoleic acid (LA) metabolite synthesised by 15-LOX-1, is another fatty acid derivative exerting protective and anti-tumourigenic role. Studies on rat skin or colon tumours indicate that 13-HODE may inhibit proliferation and induces apoptosis [17]. Another linoleic acid derivative, 9-hydroxyoctadecadienoic acid (9-HODE), produced on the 5-LOX pathway was described to stimulate cell proliferation [18]. Despite developing knowledge of various fatty acid LOX metabolite activities, there is still little information on the impact of diet and dietary fat on them, especially in tumour conditions. Fish oil containing diet significantly decreased 12-HETE, 15-HETE and 13-HODE levels in azoxymethane-induced rat colon tumours, comparing to animals consuming corn oil containing diet [19]. That correlated with enhanced apoptotic index in the fish oil containing group. Similar observation was made by Rose et al. who described significant reduction of arachidonic acid level as well as its LOX metabolites, 12- and 15-HETE, in breast tumours developed in mice fed EPA or DHA containing diet, when compared to linoleic acid high diet [20]. The authors explained inhibitory effects of dietary fish oil on breast cancer growth and metastasis with mechanisms that probably involved suppression of tumour eicosanoid biosynthesis. Ten-week supplementation of the human diet with fish oil has been shown to result in decreased production of 5-HETE and leukotriene B4 by inflammatory cells [21]. In another experiment Espada et al. observed that chia oil rich in n-3 fatty acids (63% α-linolenic acid and 21% linoleic acid) decreased 12-HETE level in murine mammary gland adenocarcinomas, compared to safflower oil (76% linoleic acid and 0.24% α-linolenic acid) and control diet (12% linoleic acid and 3.5% α-linolenic acid) in mice [22]. Ramsden et al. noted that lower dietary linoleic acid levels reduced the concentration of 13- and 9-HODE [23]. It was also observed that in breast cancer cells, trans-10,cis-12 CLA decreased cell growth and the production of hydroxyeicosatetraenoic acid (5-HETE), which is an arachidonic acid metabolite, synthesised by 5-lipoxygenase (5-LOX) [24].

The aim of this study was to establish if commercially available in pharmacies or health food stores conjugated linoleic acids mixture (cis-9,trans-11 isomer and trans-10,cis-12 isomer in equal proportions), supplemented daily after carcinogenic agent administration influenced the concentration of linoleic and arachidonic acid derivatives in rat serum and 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumours. The correlations between polyunsaturated fatty acids (PUFA) and HETE contents in serum were also investigated.

2. Materials and methods

2.1. Standards and chemicals

Eicosanoid standards: 15-hydroxyeicosatetraenoic acid (15-HETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 5-hydroxyeicosatetraenoic acid (5-HETE), 13-hydroxyoctadecadienoic acid (13-HODE), 9-hydroxyoctadecadienoic acid (9-HODE) and prostaglandin E_2 (PGE_2) were purchased from Cayman Chemical Company, USA. LC-MS grade methanol, acetonitrile, ethanol and solid-phase extraction (SPE) cartridges (Bakerbond C18, 500 mg/3 mL) were purchased from J.T. Baker. Formic acid and 7,12-dimethylbenz[a]anthracene (DMBA) were purchased from Sigma-Aldrich. Deionised water was purified on the water purification system (Direct Q, Millipore). Bio-C.L.A. was obtained from Pharma-Nord, Denmark.

2.2. Animals and experiment

Female Sprague–Dawley rats (n = 50), purchased from Division of Experimental Animals, Department of General and Experimental Pathology (Medical University of Warsaw, Poland), were used in the study. The animals (30 days old) were fed the rat standard diet (Labofeed H, Feed and Concentrates Production Plant, A. Morawski, Kcynia, Poland) ad libitum. The diet was composed of the following compounds (per 1 kg): protein (222 g), fat (50 g), fibre (45 g), ash (60 g), carbohydrates (500 g), vitamin A (15,000 IU), vitamin D3 (1000 IU), vitamin E (90 mg), vitamin K3 (3 mg), vitamin B1 (21 mg), vitamin B2 (16 mg), vitamin B6 (17 mg), vitamin B12 (80 μg), pantothenic acid (30 mg), folic acid (5 mg), nicotinic acid (133 mg), Ca (9.5 g), P (7.7 g), Mg (3 g), K (10 g), Na (2 g), Cl (2.5 g), S (1.9 g), Fe (170 mg), Mn (68 mg), Zn (78 mg), Cu (16 mg), Co (0.3 mg), I (0.2 mg), and Se (0.4 mg). Linoleic acid was the main fatty acid in the rat chow. Its concentration was about 40%, followed by α-linolenic acid (22%), oleic acid (16%), palmitic acid (13%) and stearic acid (3%). Other fatty acids were in trace concentrations. Since the 50th day of life the rats were given intragastrically 0.15 mL/day of oil rich in linoleic acid (groups A1 and G1) or Bio-C.L.A. (Pharma-Nord, Denmark) (other groups) in two concentrations. Groups B1 and D1 were given 0.15 mL/day of Bio-C.L.A., which corresponded with 1% content in a diet, whereas groups C1 and E1 were given 0.30 mL/day, equal to 2% CLA in a diet [25]. Dietary groups numbered 8 (A1, G1, D1 and E1) or 9 (B1 and C1) animals. Fatty acid profiles (%) in administered oils are introduced in Fig. 1. When 50 days old, the animals from groups A1, B1 and C1 were administered intragastrically a single dose (80 mg/kg body weight) of carcinogenic agent — 7,12-dimethylbenz[a]anthracene (DMBA, Sigma-Aldrich) to induce tumours. Fifteen weeks after DMBA administration, the rats were decapitated. Blood and tumours were collected. Serum was obtained by centrifugation of blood at 4 °C at 3000 rpm. Serum and tumours were stored at −20 °C until further analyses.

The experiment was approved by The Ethical Committee on Animal Experiments at the Medical University of Warsaw.

2.3. HETE and HODE determination

2.3.1. Sample preparation

Methanol (0.5 mL) was added to serum samples (0.4 mL), followed by water (4 mL), so as to obtain approximately 10% methanol. Tumour samples (approximately 0.2 g) were homogenised in water (2 mL). During that action the homogeniser was kept in crushed ice. The samples were incubated at 4 °C for 30 min then tumour samples were centrifugated at 3000 rpm for 5 min to remove precipitated proteins. Tumour supernatants and serum samples were loaded onto SPE

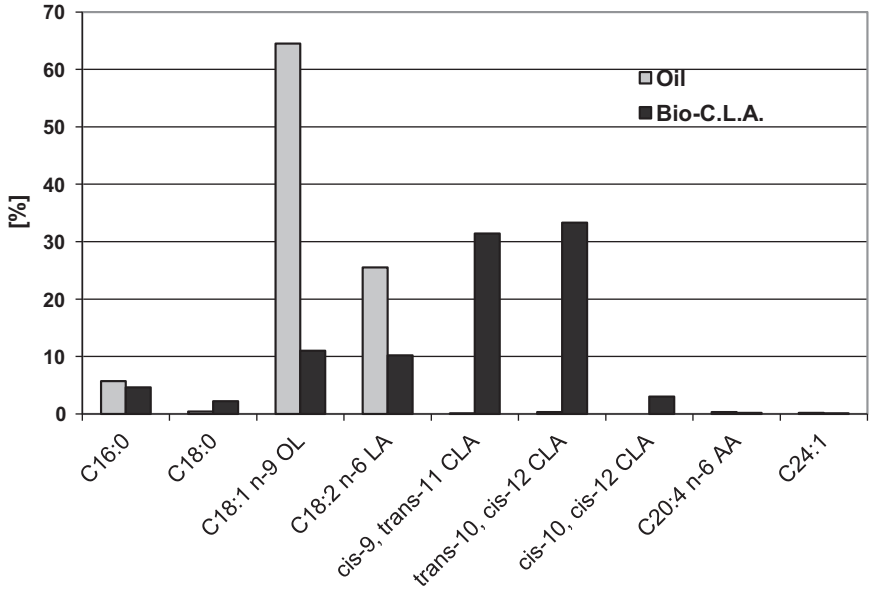

Fig. 1. Fatty acid profiles (%) in administered oils.

cartridges, which had been conditioned beforehand with 10 mL methanol followed by 10 mL water. The cartridges were washed with 2 mL water and 2 mL 10% methanol. The fatty acid metabolites were eluted with 100% methanol (3 × 0.5 mL) and evaporated to dryness in a nitrogen stream at 37 °C. The dry residues were reconstituted in 200 μL mixture of ethanol and water (1:2, v/v) and filtered through 0.22 μm filters (Ultrafree-MC, Durapore PVDF, 0.22 μm, Millipore, USA) [26].

2.3.2. LC-MS/MS analysis

The analysis of linoleic and arachidonic acid metabolites present in serum and tumour tissue was carried out using modified method by Masoodi et al. [27,28]. Chromatographic separation was performed on Dionex UltiMate 3000 HPLC system, on C18 column (Luna 5 μm, C 18(2), 150 × 2 mm, Phenomenex, USA) with guard column packed with the same material. The flow rate was 0.5 mL/min for 12 min, then it was increased to 1 mL/min. The column temperature was maintained at 30 °C. The injection volume was 100 μL. The analysis was performed in a gradient system, where the mobile phase was composed by mixing two solvents: A (water with 0.2% formic acid) and B (acetonitrile/methanol, 75:25, v/v with 0.2% formic acid). The percentage content of solvent B in the mobile phase volume was 40% from 0.0 to 1.0 min, increased to 90% between 1.0 and 10 min and maintained to 12 min. Then the content of solvent B was decreased to 40% between 12.0 and 12.1 min. This concentration was maintained to 14 min.

Eicosanoids were quantified with 3200 Q-Trap mass spectrometer (AB Sciex, Framingham, USA) operating in negative ion mode, and multiple reaction monitoring (MRM) method was performed. MS conditions were: curtain gas: nitrogen (CUR = 20), source temperature: 600 °C, ion spray capillary voltage: −4.500 V, dwell-time: 50 ms. MRM transitions and collision energy settings are summarised in Table 1 [28]. The method described enabled good separation as well as fast (14 min) analysis of compounds, which very low levels were detected. The method is characterised by good precision and recoveries. The exact validation parameters were described previously [28]. The precision, expressed as the relative standard deviation of the mean (RSD%), ranged from 5% (9-HODE) to 13% (15-HETE). Recoveries of eisosanoids were within 77% (9-HODE)–137% (5-HETE) range.

2.4. HETE and HODE correlations with CLA

2.4.1. CLA determination

CLA determination was performed with gas chromatography (GC), using capillary column and flame-ionization detector. The procedure was precisely described by Białek et al. [25].

2.5. Statistical analysis

Statistica 10.0 software (StatSoft, USA) was used for the statistical analysis. The normality assumptions were checked with Shapiro–Wilk's test. ANOVA, followed by Tukey's range test with unequal sample size, was used to compare differences between groups if the normality and variance homogeneity assumptions were fulfilled. Otherwise nonparametric Kruskal–Wallis test with post-hoc Dunn's test was performed. The acceptable level of significance was established at $p < 0.05$. Spearman's coefficients were used to estimate potential correlations between circulating CLA isomers (cis-9,trans-11 CLA and trans-10,cis-12 CLA) and linoleic and arachidonic acid metabolites. Data are presented as means ± SEM.

Fisher's exact test was used to compare tumour incidence among groups. The power of the study was over 80%, with 95% confidence interval.

Table 1
Multiple reaction monitoring (MRM) for the LC/MS/MS analysis of selected eicosanoids.

Compound	MRM (m/z)	Collision energy (eV)
PGE_2	351.1 → 271.2	−22
13-HODE	295.1 → 195.3	−24
9-HODE	295.2 → 171.0	−26
15-HETE	319.1 → 219.0	−16
12-HETE	319.2 → 179.0	−18
5-HETE	319.1 → 114.9	−18

Table 2
Mammary tumours in DMBA-treated rats according to dietary groups.

Dietary group	Supplementation	Total rat number	Tumour incidence at necropsy (%)	Tumour weight ranges (g)
A1	Oil	8	88[a]	0.45–11.93
B1	1% CLA	9	67	0.62–8.51
C1	2% CLA	9	33[a]	1.12–4.48

[a] Statistically significant differences at $p < 0.05$ were calculated with Fisher's exact test.

3. Results

The number of tumour-bearing rats in A1 and B1 groups was reported by Białek et al. [25]. In the present paper it is shown in Table 2, complemented with C1 group. The highest tumour induction was observed in A1 group (88%), supplemented with oil. The tumour incidence in groups supplemented with CLA was lower: 67% in B1 group fed 1% CLA and 33% in C1 group, fed 2% CLA.

3.1. HETE, HODE and PGE_2 determination

In our study, we determined 6 eicosanoids, metabolites of linoleic acid (13- and 9-HODE) and arachidonic acid (15-HETE, 12-HETE, 5-HETE and synthesised by COX–PGE_2) in rat serum and tumours. We also analysed statistically ratios of various eicosanoids having opposite effects in carcinogenesis: 12-HETE/15-HETE, 5-HETE/15-HETE, 12-HETE/13-HODE and 5-HETE/13-HODE.

5-HETE was the main hydroxy fatty acid extracted from serum of all dietary groups, following by 9-HODE, 12-HETE and 13-HODE, whereas 15-HETE showed the lowest levels (Table 3). We observed significant differences in the concentrations of hydroxy fatty acids extracted from serum of rats fed various diets. In the non-DMBA-treated groups (G1, D1, E1) the lowest levels of eicosanoids were observed in D1 group, fed 1% CLA (Table 3). Statistically significant differences were between D1 and A1, as well as between D1 and C1 groups. The lowest serum concentrations of LA and AA metabolites were noted in A1 group, fed with oil (Table 3). They differed significantly from CLA fed groups — B1 and C1. However, the contents of HETE and HODE extracted from B1 group were significantly increased when compared to the C1 group (Table 3). PGE_2 was undetectable in rat serum of all dietary groups, both DMBA-treated and non-DMBA-treated.

Serum eicosanoid ratios were significantly higher ($p < 0.05$) in A1 group (12-HETE/15-HETE (6.7), 5-HETE/15-HETE (20.8), 12-HETE/13-HODE (2.9) and 5-HETE/13-HODE (9.3)) compared to B1 (2.4, 6.1, 1.2, 3.0 respectively) and C1 groups (4.0, 5.2, 0.8, 1.1 respectively) (Fig. 2). 5-HETE/15-HETE, 5-HETE/13-HODE and 12-HETE/13-HODE ratios were slightly increased in B1 group compared to C1 group (Fig. 2). There were few if any differences in eicosanoid ratios in serum of the animals from non-DMBA-treated groups (data not shown).

B1 group showed the highest level of all metabolites in tumour tissues (Table 4). They were significantly higher compared to A1 and C1 groups. Similarly to results received from serum, LA and AA metabolite concentrations in A1 and C1 groups did not differ significantly, except 12-HETE level, which was statistically increased in A1 as compared to C1 group. Unlike in serum, 12-HETE contents were lower in tumours from every dietary group than other AA and LA metabolites.

PGE_2, not detected in serum, was detected in tumour tissues (Table 4). The highest concentration of that eicosanoid was determined in B1 group (1119 ng/g tissue). It differed significantly from A1 (153 ng/g tissue) and C1 (103 ng/g tissue) groups. The contents of PGE_2, which is AA COX metabolite, were also lower than AA LOX derivatives — 15-, 12- and 5-HETE in A1 and C1 groups.

Similarly to serum, 12-HETE/15-HETE and 12-HETE/13-HODE ratios were significantly higher in A1 group (0.81 and 0.92 respectively) compared to B1 (0.62 and 0.47 respectively) and C1 (0.41 and 0.39 respectively) groups (Table 4). The other two analysed hydroxy fatty acid ratios (5-HETE/15-HETE and 5-HETE/13-HODE) were also higher in A1 group than in B1 and C1 groups, although the differences were not significant (Table 4). There were slight if any differences in eicosanoid ratios between B1 and C1 groups.

3.2. HETE and HODE correlations with CLA

We found that AA metabolite levels (HETE) negatively correlated with CLA circulating in serum. At $p < 0.05$ significant correlations were noted especially between trans-10,cis12 CLA and 15-HETE ($r = -0.60$), 12-HETE ($r = 0.59$), and 5-HETE ($r = 0.71$) (Table 5).

4. Discussion

Diet plays a critical role in the aetiology of cancers and dietary fat and particular fatty acids attract the interest of researchers [29,30]. CLA has taken a special place among other fatty acids since Pariza et al. discovered anti-tumourigenic activity of fried beef in 1979, and some years later a compound responsible for that action [31,32]. The discovery caused CLA to be regarded as functional food [33]. Despite many epidemiological studies and animal experiments [34,35], the mechanisms of anticancer activity of CLA are still disputed.

In our experiment, we made an attempt to estimate the influence of CLA mixture, commercially available in pharmacies or health food stores, supplemented daily in two concentrations (1% or 2%) after carcinogen administration on the contents of LA and AA metabolites in rat serum and chemically induced tumours. We also wanted to check the possibility of using these metabolites as markers in carcinogenesis.

In the similar studies two models of CLA preparations have been used. One of them is based on a mixture containing usually equal amounts of two major CLA isomers (cis-9,trans-11 CLA and trans-10, cis-12 CLA) [36]. In the other one the focus on effects of separate CLA isomers has been put forward [37,38]. In some experiments mammary tumour development, mammary epithelial mass and size of the terminal end buds were reduced when rats fed both CLA isomer mixture or one of the CLA purified isomers [39]. On the other side, studies with purified CLA isomers (cis-9,trans-11 CLA and trans-10,cis-12 CLA) fed to laboratory animals or given to the cancer cell lines indicated that they may exert differential effects on carcinogenesis of some types of tumours as well as on fatty acid profile in various tissues [40]. In the light of that result, some authors suggested that using a single CLA isomers "is more appropriate than their equal-amount mixture", because it enables better observation of CLA anticancerogenic mechanisms [41]. Despite of that advantage of separate CLA isomers, after thorough investigation of the CLA preparations, commercially available in pharmacies or healthy food stores in some European Union countries, we decided to choose the mixture of isomers, rather than separate isomers. Such decision brings some limitations in observation of CLA mechanisms of activity. However, it enabled us to provide similar conditions to those of humans taking CLA supplements.

The lowest tumour incidence was noted in C1 group supplemented with 2% CLA (33%), followed by B1 group fed 1% CLA (67%) (Table 2).

Table 3
Concentrations of linoleic and arachidonic acid metabolites in serum of DMBA-treated or non-DMBA-treated rats supplemented with CLA or oil [ng/mL serum].

Group	A1	B1	C1	G1	D1	E1
Hydroxy fatty acid	+DMBA			−DMBA		
13-HODE	22.0 ± 4.9^{ab}	435.8 ± 219.7^{a}	233.8 ± 60.8^{b}	240.9 ± 73.2^{c}	37.5 ± 7.2^{cd}	341.4 ± 92.0^{d}
9-HODE	46.6 ± 10.4^{a}	1002.0 ± 632.9^{a}	463.2 ± 119.4^{a}	388.2 ± 84.8^{cd}	74.7 ± 15.2^{cd}	621.3 ± 240.9^{d}
15-HETE	9.4 ± 1.9^{a}	196.7 ± 80.3^{ab}	49.1 ± 23.3^{b}	139.3 ± 45.7^{c}	19.0 ± 4.8^{cd}	129.6 ± 39.7^{d}
12-HETE	61.5 ± 8.4^{a}	455.7 ± 164.3^{ab}	166.3 ± 57.0^{b}	468.0 ± 265.7^{c}	76.6 ± 12.7^{cd}	402.2 ± 265.7^{d}
5-HETE	195.8 ± 42.2^{a}	1074.0 ± 155.4^{ab}	221.0 ± 43.4^{b}	1032.4 ± 238.6^{c}	99.5 ± 41.7^{cd}	1006.7 ± 252.7^{d}

Data are expressed as mean ± SEM. Values sharing a letter (a, b, c or d) in one row are significantly different ($p < 0.05$). A value from the +DMBA group (A1, B1, C1) was compared to the other values of +DMBA groups and a value from the −DMBA group (G1, D1, E1) was compared to the other values of −DMBA groups.

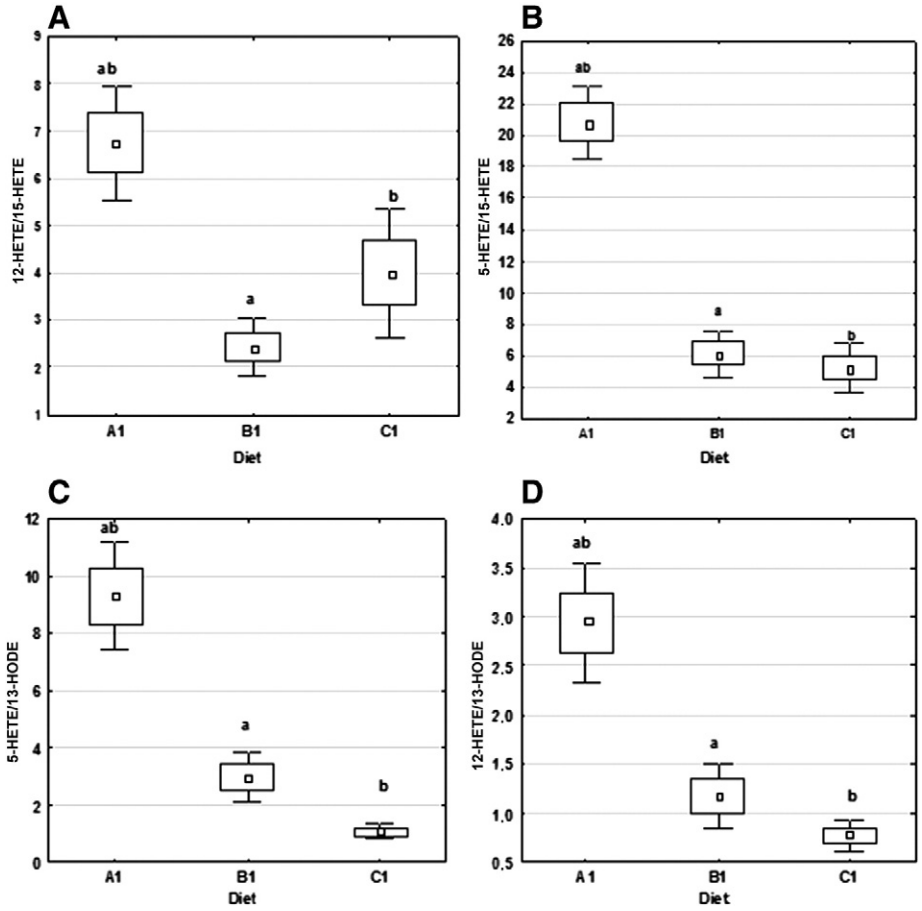

Fig. 2. Serum eicosanoid ratios in DMBA-treated dietary groups. A — 12-HETE/15-HETE ratio, B — 5-HETE/15-HETE ratio, C — 5-HETE/13-HODE ratio, and D — 12-HETE/13-HODE ratio. Values sharing a letter (a or b) are significantly different ($p < 0.05$). Small square inside large box — mean, large box — standard error of mean (SEM), whiskers — confidence level.

A1 group showed the highest tumour occurrence (88%). The obtained results confirmed the knowledge of anti-tumourigenic CLA activity [8,42]. That indicates that higher doses of CLA are more efficient in inhibition of breast cancer.

Unexpectedly, in our study we noted the highest levels of LA and AA metabolites in B1 group supplemented with lower (1%) CLA dose, followed by C1 group, where tumour incidence was decreased by 21% and 55% respectively, when compared to A1 group. The animals from A1 group were fed the oil rich in linoleic acid (Fig. 1). However, the eicosanoid contents in that group were the lowest. On the other hand, the rats in that group developed the highest number of tumours (88% incidence). That was surprising because linoleic acid is a substrate for AA synthesis by delta-6- and delta-5-desaturases and elongases. Arachidonic acid is further converted to hydroxyeicosatetraenoic acids or to PGE_2. 12-HETE and 5-HETE show tumourigenic and metastatic activity and we could expect higher concentrations of these compounds. Nevertheless, that result may be explained by Rett and Whelan [43]. Reviewing the literature, they observed: "there are no evidence to suggest that increased intake of LA would modify tissue AA content" and that may cause low eicosanoid concentrations [43].

Several studies suggested that trans-10,cis-12 CLA reduced synthesis of 5-HETE but not 12- and 15-HETE in mouse mammary tumour cells [24,44]. However, other studies present contrary results. Urquhart et al. observed that the lower concentrations of cis-9,trans-11 CLA and trans-10,cis-12 CLA isomers (50 μM) individually inhibited the overall prostaglandin production but tested at a high levels (100 μM) only cis-9,trans-11 CLA demonstrated that action. Trans-10,cis-12 CLA in a high concentration exerted pro-inflammatory activity and caused stimulation of prostaglandins [45]. In the present study we noted the most effective synthesis of eicosanoids extracted from tumours and serum

Table 4
Concentrations of linoleic and arachidonic acid metabolites in tumour tissue [ng/g tissue].

Hydroxy fatty acid	A1	B1	C1
13-HODE	785.2 ± 182.4^{a}	1888.7 ± 381.2^{ab}	714.0 ± 165.3^{b}
9-HODE	613.6 ± 179.9^{a}	1509.0 ± 221.0^{ab}	645.4 ± 180.9^{b}
15-HETE	738.0 ± 212.3^{a}	1292.9 ± 147.1^{ab}	677.5 ± 191.2^{b}
12-HETE	654.0 ± 235.7	773.7 ± 137.3^{a}	276.2 ± 74.4^{a}
5-HETE	406.5 ± 218.7	1154.4 ± 208.0^{a}	472.0 ± 151.4^{a}
PGE_2	153.0 ± 45.9^{a}	1119.2 ± 235.0^{ab}	103.2 ± 48.7^{b}
12-HETE/15-HETE	0.81 ± 0.23^{a}	0.62 ± 0.10	0.41 ± 0.09^{a}
5-HETE/15-HETE	0.73 ± 0.12	0.70 ± 0.15	0.70 ± 0.16
12-HETE/13-HODE	0.92 ± 0.37^{a}	0.47 ± 0.09	0.39 ± 0.10^{a}
5-HETE/13-HODE	0.72 ± 0.16	0.58 ± 0.17	0.66 ± 0.15

Data are expressed as mean ± SEM. Values sharing a letter (a or b) are significantly different ($p < 0.05$).

Table 5
Correlations between circulating CLA isomers and linoleic and arachidonic acid metabolites.

Correlation	Correlation coefficient (r)	P value
13-HODE vs. cis-9,trans-11 CLA	0.13	0.598
13-HODE vs. trans-10,cis-12 CLA	−0.18	0.499
9-HODE vs. cis-9,trans-11 CLA	−0.07	0.779
9-HODE vs. trans-10,cis-12 CLA	−0.47	0.064
15-HETE vs. cis-9,trans-11 CLA	−0.13	0.606
15-HETE vs. trans-10,cis-12 CLA	**−0.60**	**0.018**
12-HETE vs. cis-9,trans-11 CLA	−0.17	0.510
12-HETE vs. trans-10,cis-12 CLA	**−0.59**	**0.016**
5-HETE vs. cis-9,trans-11 CLA	−0.30	0.226
5-HETE vs. trans-10,cis-12 CLA	**−0.71**	**0.002**

Statistically significant p values ($p < 0.05$) are in bold.

collected from the rats in B1 group. The results from C1 group supplemented with 2% CLA were similar to those obtained from A1 group, except 12-HETE levels. The similar results were described by Comba et al., where 12-HETE concentration was lower in mammary tumours in mice from the group fed a diet of the highest LA concentration [46].

The highest concentrations of PGE_2 were also detected in tumours from B1 group, where cancer incidence was 67%. PGE_2 is a well-known cancer mediator, playing an important role in cell proliferation, survival, migration and angiogenesis of various types of tumours. Its elevated concentrations have been detected in breast, prostate, colon, pancreatic, lung or ovarian cancers [47–49]. PGE_2 production was also increased in the childhood neuroblastoma, where the compound enables tumour cell survival and proliferation [50]. Contrary, Lalier et al. observed that increased intracellular PGE_2 levels induced apoptosis of colon cancer cells, dependent on the expression of anticancer protein Bax [51]. These results highlight the complex role of PGE_2 in carcinogenesis. The results of present experiment corresponds with our previous results, as well as those by Sasaki et al., indicating that PGE_2 may be one of the agents affecting tumourigenesis, but it is not of the crucial importance [52,53].

Our results suggest that values of chosen LA and AA metabolites are not very precise factors for reflecting dietary CLA influence on the organism in pathological (cancer) conditions. For that reason the comparison of various hydroxy fatty acid ratios appears interesting and important. We compared 12-HETE/15-HETE, 5-HETE/15-HETE, 12-HETE/13-HODE and 5-HETE/13-HODE ratios, which are also used by other authors [46]. Whereas there were few if any differences in eicosanoid ratios in serum of non-DMBA-treated groups, we found interesting relationships between them, both in serum and tumours of DMBA-treated rats. Hydroxy fatty acid ratios were significantly higher in serum obtained from the rats from A1 group supplemented with LA rich oil and developing the largest number of tumours, than those from B1 and C1 groups, fed with 1% or 2% CLA, respectively. Cancer incidence in that group is also lower. What is more, except 12-HETE/15-HETE, the ratios were lower in C1 group (the lowest tumour incidence), although the differences were not significant (Fig. 2). Eicosanoids chosen to those ratios exert opposite actions. 5-HETE and 12-HETE stimulate tumourigenesis and metastasis. 15-HETE and 13-HODE act adversely [12,17].

12-HETE/15-HETE and 12-HETE/13-HODE ratios appear to be of great interest in tumour tissue. The lower values of the eicosanoid ratios in tumours from B1 and especially C1 group correlate with decreased tumour incidence in these groups. The similar relationship was described by Comba et al., who noted the highest 13-HODE/12-HETE (which means the lowest 12-HETE/13-HODE) ratio in tumours from the group of the decreased metastasis number and increased survival [46].

Another interesting fact resulting from our study is the correlation between circulating CLA isomers and HETE and HODE concentrations in serum. We found strong negative correlations between trans-10,cis-12 CLA and 15-, 12- and 5-HETE. This result corresponds partially with experiments described by Kim et al., where trans-10, cis-12 CLA diminishes 5-HETE level in human breast cancer cells [24].

5. Conclusions

In summary, the results of our study showed that CLA daily supplementation after carcinogen administration influenced LA and AA metabolite levels in serum and tumours. However, the ratios of eicosanoids having opposite effects, not concentrations of particular compounds, appear to be better indicators of pathological processes. Our experiment confirms how various and difficult is tumourigenesis and the impact of diet on it remains questions requiring further investigations.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MJ participated in the study design, carried out the experiment, performed statistical analysis and wrote the manuscript, AB designed the study and carried out the experiment, HM and IG carried out LC-MS/MS analysis, AT coordinated the study.

All authors read and approved the final manuscript.

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